On the role of calcium in normal and impaired insulin secretion

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10.1 GENERAL DISCUSSION AND SUMMARY

It is well known that exposure of islets to high glucose concentrations causes movement of secretory granules in the B-cells towards the plasma membrane and fusion of the secretory granule membranes with the plasma membrane. Thereafter, the fused membranes break and the content of the granules is released into the extracellular space (32, 33). It is thought that a rise of the cytosolic Ca^{2+} concentration triggers the exocytosis of secretory granules. This assumption is based on the following observations:

1. Sustained insulin release by isolated islets is elicited by glucose only when a sufficient concentration of extracellular calcium is present. When during glucose-stimulated insulin release the extracellular calcium concentration is decreased to a low level (less than 0.1 mM) the secretion rate rapidly declines to a low value (47). This indicates that a certain concentration of the cation is essential for the proper functioning of the secretory machine. Possibly, Ca^{2+} is required for the secretory events at the cell membrane or for maintenance of an intracellular Ca^{2+} pool with a high turnover rate, which controls insulin secretion.
2. A direct regulatory role of calcium is indicated by the observation that calcium ions itself can trigger insulin secretion. An increment of the extracellular Ca^{2+} concentration from 0 or 2.6 mM to 13.5, 20.5 or 41 mM in the absence or presence of a low glucose concentration stimulates insulin secretion (10, 11, 18). The secretory response to calcium is inhibited when the calcium uptake by the B-cells is inhibited (11). This supports the view that Ca^{2+} accumulation at some site in the B-cell is sufficient to trigger insulin secretion.
3. This view is also supported by the observation that the ionophore A23187 in the absence of glucose provokes a strong temporary increase of insulin release in the presence of extracellular Ca^{2+} but not in the absence (48). Moreover, when the Ca^{2+} uptake is inhibited by verapamil the ionophore does not cause insulin release (48).
4. Electrophysiological studies have shown that stimulation of islets with glucose causes action potentials in the B-cells, which are thought to be due to Ca^{2+} entry in the B-cells (37).
5. From electron microscopic studies employing pyroantimonate for visualization of calcium it has been concluded that glucose stimulation causes an intracellular accumulation of calcium (22, 30, 44).
6. Stimulation of insulin secretion by glucose increases ^{45}Ca uptake by

islets (20). All other insulinotropic agents so far investigated for this purpose (hexoses, glyceraldehyde, amino acids, cyclic AMP, theophylline, potassium ions, sulfonylureas) affect calcium handling, which suggests that these agents exert their stimulant action upon insulin secretion by causing calcium accumulation in the B-cell (4, 17, 20, 31, 46).

Glucose and other initiators of insulin secretion are supposed to promote the accumulation of Ca^{2+} in a cytoplasmic pool. This pool is regulated by processes in the plasma membrane. However, the cytoplasmic Ca^{2+} concentration may also be influenced by changes in the intracellular distribution of calcium. Labelling of islets with ^{45}Ca followed by fractionation and measurement of the intracellular distribution of radioactivity indicates that the secretory granules and mitochondria contain significant amounts of exchangeable calcium subject to regulation by glucose and cAMP (17, 19, 31). For the time being, we do not know exactly how Ca^{2+} triggers insulin secretion.

Recent studies have shed some light on the role of free Ca^{2+} in stimulation of insulin release. Watkins & Cooperstein (51) studied the binding of islet secretory granules to plasma membrane vesicles. Free Ca^{2+} (10^{-7} - 10^{-4}M) caused calcium concentration-dependent and calmodulin-dependent increase of the binding of secretory granules to the plasma membrane vesicles (only inside-out vesicles). The results suggest that Ca^{2+} in the presence of calmodulin modulates the interaction between the granules and the plasma membrane.

The arguments given above make it very likely that a rise of the cytoplasmic Ca^{2+} concentration triggers insulin secretion. However, it is unclear by which mechanism glucose enhances the ionized calcium concentration in the B-cells, and the origin of this calcium fraction.

The present study aimed to elucidate the latter questions by investigating:

1. The effects of glucose on islet calcium uptake, total islet calcium content, and on the physico-chemical state of islet calcium.
2. The effects of fasting (model for impaired insulin secretion) on islet calcium metabolism.

10.2 METHODS

Total calcium content of B-cells of islets of Langerhans in situ was determined by electron-probe micro-analysis (chapter 3). Pancreatic tissue and isolated islets were dehydrated by freeze-substitution. The total calcium

content of isolated islets was determined by a newly developed fluorometric micromethod (chapter 4). A histochemical method, using GBHA (= glyoxal-bis-2-hydroxyanil), was developed to stain ionized (possibly also readily ionizable) calcium (chapter 2). The population density of B-cell granules was determined by staining with aldehyde fuchsin (chapters 2, 3 and 6). GBHA-Ca and the aldehyde fuchsin positive granulation was assessed by scanning the absorbance of the B-cells with a cytophotometer. Calcium uptake was measured with ^{45}calcium.

10.3 TOTAL CALCIUM AND GBHA-Ca CONTENT OF PANCREATIC TISSUES

There is a remarkable difference in the total calcium content of both secretory tissues of the pancreas. The B-cells of islets of Langerhans have a high calcium content; 2-3 times as high as the exocrine tissue (chapter 3). The total calcium content of islet tissue and exocrine tissue amounts to 31 $\pm$ 3 and 10 $\pm$ 0.5 pmol per µg dry weight (chapter 5). The total calcium content of exocrine tissue per unit of dry weight is comparable to that of liver, kidney and muscle cells (1). Staining of both tissues with GBHA reveales that B-cells contain high and exocrine tissue very low concentrations of GBHA-Ca (chapter 3). The GBHA-Ca content of B-cells is estimated to be about 40% of the total calcium content (chapter 4).

Fasting for 24-72 h decreases the GBHA-Ca content of the B-cells by 55-60% without altering the total calcium content of the B-cells (chapter 3). Strong stimulation of insulin secretion by repeated administration of high doses of tolbutamide causes an almost disappearance of GBHA-Ca. In this case the total calcium content of the B-cells decreases by 35%. Tolbutamide treatment does not alter the total calcium content of exocrine tissue (chapter 3). The results indicate that calcium in the B-cells exists in two states: GBHA detectable and GBHA undetectable which is interpreted as ionized and unionized calcium. Fasting causes a shift to unionized calcium (chapter 3).

10.4 LOCALIZATION OF GBHA-CALCIUM

All GBHA-Ca is localized intracellularly as removal of extracellularly bound calcium by incubation of isolated islets with lanthanum (La^{3+}) does

not alter the amount of GBHA-Ca (chapter 8). Gradually decreasing the content of secretory granules of the B-cells by oral tolbutamide administration to the rats is associated with a gradual decrease of the GBHA-Ca content. After cessation of the treatment the B-cells regranulate and the GBHA-Ca restores more or less parallel to the AF-positive granulation (chapter 6). Subcellular fractionation of islets and staining of the granules reveales that the granules contain GBHA-Ca. Purified islet mitochondria are difficult to obtain, however, mitochondria from the liver do not stain with GBHA (unpublished observations). *These results indicate that calcium detectable by GBHA is mainly (or almost completely) localized in the secretory granules of the B-cells.*

10.5 EFFECT OF GLUCOSE STIMULATION AND CALCIUM MANIPULATION ON ^{45}CALCIUM UPTAKE, GBHA-Ca AND TOTAL CALCIUM CONTENT OF ISLETS OF FED RATS

Calcium metabolism of incubated isolated islets was investigated. The results indicate:

1. GBHA-Ca, an ionized calcium fraction in the secretory granules, is very mobile.

2a. Glucose (15 mM) stimulation of islets in the presence of 2.5 mM calcium causes a rapid decrease (40%) of GBHA-Ca, followed by a rise after 30 min to levels above the initial value (chapters 4 and 7).

2b. Glucose stimulation does not alter total islet calcium content (chapter 5).

2c. Upon incubation with ^{45}Ca islets exchange in the presence of 2.5 mM glucose in 5 and 30 min 21 and 28% of their calcium, respectively. With 15 mM glucose in the medium 30 and 45% of islet calcium is exchanged in 5 and 30 min, respectively. Glucose stimulation enhances the calcium-exchangeable pool (chapters 5 and 7).

3a. When extracellular calcium is decreased from 2.5 mM to 43 µM the GBHA-Ca response to glucose is blocked. The GBHA-Ca level is maintained at a high level at 15 mM glucose, but at 2.5 mM glucose GBHA-Ca is abruptly decreased by 40% (chapters 4 and 7).

3b. Low extracellular calcium causes a gradual decrease of total islet calcium; 20% over 30 min. The decrease of islet calcium is not significantly influenced by stimulation with 15 mM glucose (chapter 7).

3c. At low extracellular calcium the ^{45}Ca uptake is strongly decreased, but

the stimulating effect of 15 mM glucose is not abolished (chapter 7).

4a. Reintroduction of extracellular calcium (2.5 mM) restores GBHA-Ca at 2.5 mM glucose partly and at 15 mM glucose completely after which the normal decrease and secondary rise occurs (chapter 4).

4b. Reintroduction of extracellular calcium restores the total islet calcium content at 2.5 mM glucose within 15 min. The restoration is not affected by glucose stimulation (chapter 7).

4c. At 2.5 mM glucose the ^{45}Ca uptake equals the restoration of the total islet calcium content, which indicates that calcium efflux is temporary almost absent. At 15 mM glucose ^{45}Ca uptake is higher than the increase of the total islet calcium content, which is due to calcium exchange (chapter 7).

5. Degranulated islets have a very low GBHA-Ca content (chapter 6). GBHA-Ca in degranulated islets does not respond to glucose stimulation, to exposure to "calcium-free" buffer and to reintroduction of extracellular calcium (chapter 8).

Changes in glucose-induced GBHA-Ca of the secretory granules are not related to changes of the total islet calcium content. Removal of extracellular calcium blocks the glucose-induced GBHA-Ca response and insulin secretion. Reintroduction of extracellular calcium immediately restores the GBHA-Ca response and insulin secretion (chapter 4).

10.6 EFFECTS OF FASTING ON INSULIN SECRETION, GBHA-Ca, TOTAL ISLET CALCIUM CONTENT AND ^{45}Ca UPTAKE

Fasting for 24-72 h delays the insulin secretory response upon stimulation with 15 mM glucose. After 45 min insulin secretion rates of fed and fasted islets are comparable. The inhibition of insulin secretion increases with increasing periods of fasting (chapters 8 and 9). It has been shown by Malaisse-Lagae and Malaisse (36) that under a variety of experimental conditions ^{45}Ca uptake correlates with insulin release. It is assumed that glucose causes an increase in the cytosolic calcium concentration which triggers insulin secretion. The decreased secretory response of fasted islets may be due to a decreased ^{45}Ca uptake. Levy et al. (34) have observed a 50% decreased ^{45}Ca uptake over 90 min in islets of 48-h-fasted rats. However, in the present study, the ^{45}Ca uptake over 5, 30 and 60 min at 2.5 and 15 mM glucose is not altered by 24 h of fasting. Fasting for 72 h slightly decreases

the ^{45}Ca uptake at 15 mM glucose over a period of 30 min but not over a period of 60 min (chapter 9). The difference of our results with those of Levy et al. (34) may be due to the use of different techniques, since they extensively washed the islets after ^{45}Ca uptake. The total calcium content of islets is not altered by fasting for 24 h or 72 h (chapter 9). Apparently, an increased ^{45}Ca uptake by islets does not suffice to elicit insulin secretion. Therefore, factors other than an increased ^{45}Ca uptake must play a crucial role in the fasting-induced inhibition of the secretion.

One such factor may be the correct physiological intracellular distribution of Ca^{2+} after its initial entry through the plasma membrane. The measurement of GBHA-Ca of B-cells reveals that fasting alters the intracellular calcium handling (chapter 8). The GBHA-Ca content of the B-cells of fasted islets is decreased and the glucose-induced decrease and the secondary rise as seen in fed islets is completely or almost completely suppressed (chapter 8). Furthermore, when islets are washed with and incubated in "calcium-free" buffer the GBHA-Ca levels decrease to very low levels. Exposure of the calcium-depleted islets to 2.5 mM and 15 mM glucose in the presence of calcium rapidly restores the GBHA-Ca levels in fed islets but not completely in fasted islets (chapter 8). As GBHA-Ca is mainly localized in the secretory granules the results suggest that the mechanism which regulates the granular Ca^{2+} content is altered by fasting. A factor which may influence the intracellular calcium distribution is cAMP. It has been shown that cAMP causes translocation of calcium from an organelle-bound pool to the cytoplasma (3, 16, 17). In fed islets there is a rough inverse relationship between GBHA-Ca level and cAMP level during glucose stimulation (compare chapters 4 and 9 with reference 2). As fasting decreases the cAMP response we suppose that the decreased cAMP response in fasted islets is involved in the decreased response of GBHA-Ca to glucose stimulation.

10.7 POSSIBLE MECHANISM FOR GLUCOSE-INDUCED GBHA-Ca CHANGES

Several lines of evidence indicate that glucose stimulation alters the calcium content of the secretory granules. It has been shown by rontgen micro-analysis and by the pyroantimonate technique that the secretory granules and the mitochondria of the B-cells contain high concentrations of calcium (22, 24, 29, 30, 44). Incubation of islets or perfusion of pancreases with 16.7 - 20 mM glucose for 20-120 min strongly increases the calcium-

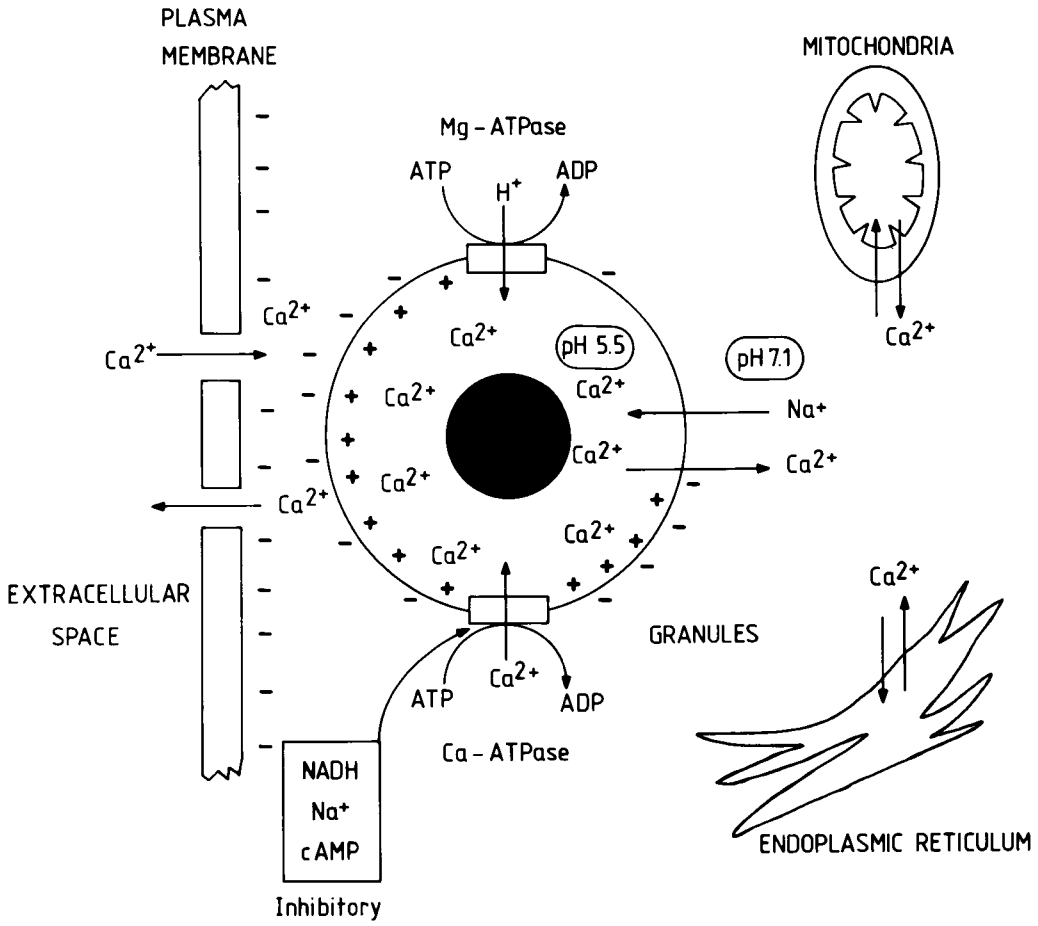

Figure 10.1

Model for regulation of cytoplasmic and granular calcium concentration of the B-cells.

pyroantimonate precipitates in the granules (22, 29, 30, 44). Glucose stimulation causes an increase of the uptake of ^{45}Ca which is mainly incorporated in the granules and the mitochondria (31). Glucose stimulation strongly increases the ^{45}Ca efflux from islets labelled at high but not from those labelled at low glucose concentrations (15). Condition with increased cAMP level in islets enhance the efflux of ^{45}Ca from the granules and inhibit the uptake of ^{45}Ca by the mitochondria (17). Furthermore, isolated granules accumulate ^{45}Ca, which process is strongly stimulated by ATP and calcium (5) and inhibited by cAMP (45). In addition the granules contain a Ca-ATPase activity which is stimulated by ATP and calcium (10^{-8} - 10^{-4} M) and inhibited by cAMP, NADH and in a concentration dependent manner by NaCl (probably Cl^- not inhibitory) (5, 13) see Fig 10.1. Inhibition of the Ca-ATPase activity may provide a simple explanation for the effect of glucose on the granular calcium content. Glucose may cause calcium release from the granules by enhancing one or more of the factors which inhibit the Ca-ATPase activity. Glucose stimulation of rat islets immediately enhances the NADH and NADPH content (35, 50). Moreover it has been shown that glucose stimulation increases the cAMP content of the islets (2). Probably, glucose stimulation also enhances the proton-translocating Mg-ATPase activity in the granule membrane (27, 39), which results in an increase of the H^+ concentration in the granules. The decrease of the pH favours the ionization of intragranular calcium. It has been shown that secretory granules of other endocrine glands exchange Ca^{2+} for Na^+ (42). Preliminary results with pancreatic islets indicate that high intracellular Na^+ decreases and low intracellular Na^+ increases the GBHA-Ca content of the islets.

In addition it has been shown that glucose (16.7 mM) stimulation for 90 min moderately decreases the sodium content of rat islets (28). Therefore we suppose that the intracellular Na^+ concentration, by means of Na^+-Ca^{2+} exchange, plays an important role in the regulation of the Ca^{2+} concentration of the surrounding cytoplasma. All conditions seem present for a glucose metabolism-dependent regulation of the granular calcium store. Therefore, it seems likely that the glucose-induced GBHA-Ca changes are achieved by the mechanism as described above. The inhibition of Ca-ATPase activity may provide a simple explanation for the glucose-induced insulin secretion. Besides the secretory granules the mitochondria and the endoplasmic reticulum also contain Ca-ATPase activity (5, 13). Furthermore, calcium fluxes across the B-cell membrane may also contribute to the cytosolic calcium concentration, which is assumed to regulate insulin secretion.

10.8 POSSIBLE OSMOTIC FUNCTION OF GBHA-Ca CHANGES

GBHA stains ionized calcium and the secretory granules contain a relatively high concentration (chapters 2, 3 and 4). The presence of ionized calcium in the granules may be due to the acidic pH, which is about 5.5 (26). This low pH is mainly generated by a proton-translocating Mg-ATPase activity in the granule membrane (27). On stimulation the proton gradient across the granule membrane increases (39), which favours the ionization of intragranular calcium and would counteract a possible conversion to unionized calcium. Efflux of ionized calcium to the cytosol, which has a pH of 7.1 (21), can explain the decrease of GBHA-Ca on glucose stimulation (see Fig 10.1).

The total islet calcium content is 31 pmol/µg dry weight (chapter 5). Assuming a dry weight/wet weight ratio of 20/100 the calcium concentration in islet cells would be 6.2 mM. The GBHA-Ca fraction is estimated to be about 40% of the total calcium content (chapter 4). Thus, overall the GBHA-Ca concentration in the cell is 2.5 mM. However, GBHA-Ca is localized in the secretory granules which account for only 11.7% of the volume (9). The secretory granule consists of a granular sack containing a crystalline core (14) (zinc-insulin complex; 12, 41), which occupies 40% (20-60%) of the volume (calculated from 23, 28, 40) and contains no calcium (29). GBHA-Ca is therefore localized in only 7% (11.7 x 0.6) of the cell volume and the concentration of GBHA-Ca is about 36 mM. On glucose stimulation 40% (see chapter 7) of GBHA-Ca disappears. This would imply that if this amount is translocated to the cytosol the granular calcium concentration would decrease by 14 mM and the cytosol would increase by 1 mM. The significance of such an enormous translocation of calcium is not clear. If the ionized calcium concentration decreases by 14 mM as a result of efflux to the cytosol it must be accompanied by a counterion e.g. Cl^- or it must be exchanged by another ion e.g. Na^+. In the first case the osmotic pressure would decrease by 42 mosmoles and in the second case increase by 14 mosmoles.

Isolated secretory granules in iso-osmotic sucrose are very sensitive to sodium (not to potassium) which ion induces lysis of the granules (6, 25). Furthermore, in the chromaffin granule membrane (adrenal medulla) which is very similar to that of the B-granule membrane a Na^+-Ca^{2+}-exchange mechanism has been shown (42). Moreover, both glucose-stimulated ^{45}Ca efflux from islets and insulin secretion are strongly inhibited by omission of extracellular sodium. Therefore, we suppose that sodium plays a role in this

mechanism.

Several lines of evidence indicate that exocytosis might be based on restricted osmotic lysis (43, 49, 52). It has been shown in an artificial system that phospholipid vesicles fuse with a planar membrane when the planar membrane contains a calcium-binding protein and when also micromolar concentrations of Ca^{2+} and an osmotic gradient are present (52). It has also been demonstrated that isolated secretory granules from islets, pituitary and adrenal medulla in hypo-osmotic medium interact with their respective isolated plasma membranes and release hormone (insulin, somatotropin, adrenalin) when 2.0 µM Ca^{2+} is added (7, 8). As already mentioned above, free Ca^{2+} (10^{-7} - 10^{-4} M) produces a concentration-dependent binding of secretory granules to plasma membranes isolated from fish islets (51). The binding was strongly dependent on the presence of calmodulin (51).

We would put forward the hypothesis that GBHA-Ca in the granules of the B-cells is involved in the regulation of the Ca^{2+} concentration of the surrounding cytoplasma, which in the presence of calmodulin modulates the interaction of the secretory granules with the plasma membrane. The changes in granular calcium would provide the osmotic gradient which causes the breakage of the fused membranes resulting in extrusion of the insulin content.